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# Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation and DNA integrity of cryopreserved bovine sperm

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´´Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation and DNA integrity of cryopreserved bovine sperm´´

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## 1. Abstract

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**Effekte von Natriumpyruvat auf die Lebensfähigkeit, Synthese von reaktiven Sauerstoffspezies, Lipidperoxidation und DNA-Integrität von kryokonserviertem Rindersperma**

In der Studie wurden die Effekte von Natriumpyruvat (Na-Pyr) auf die Lebensfähigkeit sowie die Synthese reaktiver Sauerstoffspezies (ROS), die Lipidperoxidation und die DNA-Integrität kryokonservierten Rinderspermas untersucht. Dazu wurden von 23 Zuchtstieren je drei Ejakulate ( n = 69) gewonnen, gesplittet und mit einem Tris-Eidotter-Medium ohne und mit 5 mM Na-Pyr verdünnt. Das Sperma wurde bei -196 °C eingefroren und unmittelbar nach (0 h) dem Wiederauftauen sowie nach 3, 6, 12 und 24 h Inkubation bei 37 ° C untersucht. Die Anteile lebensfähiger Spermien (sich schnell bewegend (RMS), plasmamembran- und akrosomintakt (PMAI), hohes Mitochondrienmembranpotential (HMMP)), Spermien mit hoher DNA-Fragmentation (%DFI) sowie die Ausmasse der ROS-Synthese (Dichlorfluorescein-diacetat (DCFH), CellROX Deep Red® (CellROX)) und der Lipidperoxidation (LPO) der Spermien wurden bestimmt. Mit Na-Pyr kryokonservierte Spermien hatten höhere Werte für RMS, PMAI und HMMP, CellROX und niedrigere %DFI Werte ($P < 0.001$) als Spermien ohne Na-Pyr, unterschieden sich aber nicht ($P > 0.05$) von diesen in den LPO- und DCFH-Werten. Die Ergebnisse zeigen, dass Natriumpyruvat die Lebensfähigkeit und DNA-Integrität kryokonservierten Spermas verbessert, jedoch die

Lipidperoxidation nicht beeinflusst, obwohl die Synthese bestimmter reaktiver Sauerstoffspezies zunimmt.

**Schlüsselwörter: Bullspermatozoen; DNA-Integrität; Natriumpyruvat; Reaktive Sauerstoffspezies; Wasserstoffperoxid**

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**Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation and DNA integrity of cryopreserved bovine sperm**

In this study, the effects of sodium pyruvate (Na-Pyr) on the viability as well as on synthesis of reactive oxygen species (ROS), lipid peroxidation and DNA integrity of cryopreserved bovine sperm were investigated. For this purpose, three ejaculates (n = 69) were collected from 23 AI bulls, splitted and diluted with a Tris egg yolk extender without and with 5 mM Na-Pyr. Sperm samples were frozen at -196 ° C, and examined immediately after thawing (0h) as well as after 3, 6, 12 and 24h incubation at 37 ° C. The percentages of viable sperm (rapidly motile (RMS), plasma membrane and acrosome intact (PMAI), high mitochondrial membrane potential (HMMP)), high DNA fragmentation (% DFI), ROS synthesis (dichlorofluorescein diacetate (DCFH), CellROX Deep Red® (CellROX)) and lipid peroxidation (LPO) of the sperm were determined. Cryopreserved sperm diluted with Na-Pyr showed higher values for RMS, PMAI and HMMP, CellROX and lower values for %DFI ($P < 0.001$) compared to sperm without Na-Pyr, but there was no effect ($P > 0.05$) of Na-Pyr on LPO and DCFH values. The results show that sodium pyruvate improves the viability and DNA integrity of cryopreserved sperm but did not affect lipid peroxidation, although it increased the synthesis of ROS.

**Key words: Bull spermatozoa; DNA integrity, Sodium pyruvate; Reactive oxygen species; Hydrogen peroxide**

## 2. Introduction

It is well known that the cryopreservation process induces sublethal damages to the spermatozoa, including reduction of sperm motility, disturbances plasma membrane and acrosome integrity, decrease of mitochondrial membrane potential, rise of reactive oxygen species (ROS), lipid peroxidation and damage of DNA (Bollwein et al., 2008; Januskauskas et al., 2003; Thomas, 1998).

Hydrogen peroxide ($H_2O_2$) is a ROS that is produced in sperm spontaneously (Upreti et al., 1998). Reactions catalyzed by aromatic amino acid oxidase (AAAO) and superoxide dismutase (SOD) are primary sources of $H_2O_2$ in bovine spermatozoa (Upreti et al., 1998). The Electron Transport Chain (ETC) and the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) pathway in the mitochondria are suitable places for ROS generation (Koppers et al., 2008). $H_2O_2$ can diffuse easily through the cellular membrane, react with $Fe^{2+}$ and generate hydroxyl radical (OH•) (Fenton reaction) (Ward et al., 1985). This OH• radical decreases mitochondrial mRNA output by attacking mtDNA, thus resulting in altered synthesis of mitochondrial proteins, which are essential for ETC and ATP synthesis (Jenkins et al., 2014). Furthermore, $H_2O_2$ binding to the nuclear DNA can induce single-strand breaks (SSBs) and base damages in sperm (Aitken et al., 1998; Rueff et al., 1993; Villani et al., 2010; Ward et al., 1985). In line with this, our group showed (Gürler et al., 2016) that remarkable changes in DNA damage are associated with increasing intracellular concentrations of $H_2O_2$ in cryopreserved bovine sperm. Thus, we hypothesized that an inhibition of the synthesis of $H_2O_2$ could improve DNA integrity of cryopreserved bovine sperm.

To be able to protect cryopreserved bovine sperm from oxidation and its harmful effects due to presence of oxidative stress, various antioxidants e.g. taurine (Tirpák et al., 2015), vitamin E, ascorbic acid, glutathione, cysteine, coenzyme Q10 (Rao et al., 2013), methionine, carnitine and inositol (Bucak et al., 2010) as well as sodium pyruvate (Bilodeau et al., 2002) are added to semen extenders as protective additives. Pyruvate acts as a $H_2O_2$ scavenger, which decarboxylases it to acetic acid, carbon dioxide and water without any oxygen release (Bilodeau et al., 2002; Breininger and Beconi, 2014; Giandomenico et al., 1997; Melzer and Schmidt, 1988; O'Donnell-Tormey et al., 1987; Salahudeen et al., 1991; Upreti et al., 1998). In contrast to other antioxidants, pyruvate is the end product of the glycolysis in the cellular metabolism, which is converted into acetyl-CoA and enters the Krebs cycle to produce ATP during oxidative phosphorylation (OXPHOS). A main ROS generated in spermatozoa is superoxide anion ($O_2^{\cdot-}$). This electron-reduced product of oxygen reacts with itself via dismutation to generate $H_2O_2$ (Lushchak, 2014).

Thus, minor differences of the oxygen amount could affect ROS generation and sperm cell survival. In this regard, sodium pyruvate may be preferable as an antioxidant, due to its positive effects on intracellular energy metabolism, post-thaw sperm quality (Bilodeau et al., 2002), as well as the ability to scavenge $H_2O_2$ without oxygen releasing.

The effects of addition of pyruvate as an antioxidant to boar (Breininger and Beconi, 2014), stallion (Gibb et al., 2015) and bull (Bilodeau et al., 2002) sperm have already been investigated. Its positive effects on motility, plasma membrane and acrosome integrity were reported for bovine sperm (Bilodeau et al., 2002). Additionally, lipid peroxidation, ROS synthesis and DNA integrity were described for different mammalian species (Breininger and Beconi, 2014; Gibb et al., 2015; Upreti et al., 1998). However, to the best of our knowledge up to now there is no information if pyruvate affects lipid peroxidation, ROS synthesis and DNA damage caused by the rise of ROS after freezing and thawing of bovine sperm.

Therefore, the objectives of this study were to investigate not only the viability of sperm but also the sperm characteristics mentioned above in bovine sperm cryopreserved without and with the addition of sodium pyruvate.

## 3. Material and Methods

### 3.1. Chemicals

All chemicals were obtained from Sigma-Aldrich Co (Steinheim, Germany) unless otherwise indicated.

### 3.2. Bulls

Ejaculates were collected from 23 Simmental bulls, 4.5 ± 3 years of age, maintained at the Besamungsverein Neustadt Aisch, Germany. The bulls were on a regular collection schedule, had passed a standard breeding soundness evaluation, and had produced sperm with acceptable post-thaw characteristics (progressively motile sperm > 70%) and fertility (Non Return Rate > 65%). Semen was collected twice weekly, with no apparent changes in animal health or semen quality throughout the semen collection interval.

### 3.3. Study design

Three ejaculates were collected from each of the 23 bulls (in total 69 ejaculates). After sperm dilution, ejaculates were divided into two equal aliquots. The aliquots were diluted with Triladyl® (Minitüb GmbH, Tiefenbach, Germany) without and with the addition of 5 mM sodium pyruvate (Pyr) (Bilodeau et al., 2002).

Both aliquots were immediately cooled to 4 °C, maintained at that temperature for 24 h, and then frozen. After cryopreservation, sperm samples were thawed, and examined immediately after thawing (0 h) as well as after 3, 6, 12, and 24 h incubation at 37 °C. The percentage of rapidly motile sperm (RMS) was determined with a computer assisted sperm analysis (CASA) system. Sperm quality was evaluated by measuring the percentage of plasma membrane as well as acrosome intact sperm (PMAI), the lipid peroxidation of sperm (LPO), the percentage of sperm with a high mitochondrial membrane potential (HMMP), the amount of ROS synthesis (dichlorofluorescein-diacetate (DCFH-DA), CellROX Deep Red Reagent® probe (CellROX)) and the percentage of sperm with a high degree of DNA fragmentation (%DFI) using six flow cytometric assays.

### 3.4. Semen collection, evaluation, dilution, and preservation

Semen was collected using an artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany) and a mount animal. Only ejaculates with ≥ 70% progressive motile sperm, estimated subjectively using a phase contrast microscope with × 100 magnification (Dialux 20, Leitz, Wetzlar, Germany) were used. Sperm concentration was determined using a Sperm Quality Analyzer (SQA-Vb, SION A.I. Company, Israel). Two fractions of Triladyl®

extender (Minitüb GmbH, Tiefenbach, Germany) were prepared and pre-warmed at 37 °C: a) fraction A without prior addition of antioxidant or other reagents, and b) fraction B which was enriched with 5 mM sodium pyruvate as described by Bilodeau et al. (2002). Ejaculates were splitted into two equal aliquots; the first and second aliquot of each ejaculate was diluted with fraction A (control group) or fraction B (Pyr-treated group), respectively, to a final concentration of $65\times10^6$ sperm/mL.

All sperm samples were cooled at 4 °C for 24 hours. After 24 hours, sperm were packaged in 0.25 mL French straws (IMV Technologies; L'Aigle, France) at 4 °C using a fully automatic straw filling and sealing machine (MPP Quattro, Fa. Minitub, Tiefenbach, Germany). After filling and sealing, straws were transferred to the freezing chamber and frozen horizontally in racks. Straws were frozen in liquid nitrogen vapor at −95 °C for 9 minutes (NIFA Technologies BV, Leeuwarden, Netherlands). Thereafter, frozen samples were plunged into liquid nitrogen (−196 °C) and stored at least 24 hours before analysis. Four straws were thawed and pooled for each set of analyses. Frozen samples were thawed by immersing straws in a water bath (37 °C for 30 seconds). Aliquots of the diluted semen were diluted to a concentration of $5 \times 10^6$ sperm/mL with prewarmed (37 °C) Tyrode's medium and kept at 37 °C until analyzed.

### 3.5. Computer assisted sperm analysis

The IVOS II CASA system driven by software version 14 (Hamilton Thorne Inc., Beverly, U.S.A.) was used to assess sperm motility. For the measurements, equal parts of extended semen and Tyrode’s solution were mixed and analyzed. The extended semen with Tyrode´s solution was stained with 80 µg/mL Hoechst 33342, which was used to stain sperm DNA in order to discriminate accurately between sperm and non-sperm particles (especially

egg yolk components) (Tardif et al., 1998) using the Ident Fluorescence Option "Full Analysis" of the IVOS II system. For each sample, a 20 μm-deep semen analysis Leja 4-chamber slide (Leja, Nieuw-Vennep, the Netherlands) placed on a pre-warmed stage (37 °C) was filled with semen and a minimum of 1000 cells were analyzed in no less than eight randomly selected fields, with 30 frames acquired per field at a frame rate of 60 Hz. For further analysis the percentage of RMS with an Average Path Velocity (VAP) ≥50 μm/s was used.

### 3.6. Flow cytometric analysis

Flow cytometric analyses were assessed with a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA). Semen samples were evaluated using two laser beams generated by 488 nm (50 mW laser output) and 638 nm (50 mW laser output).

Measurements of fluorescence emissions were estimated for green fluorescence emissions by using a 525±40 nm filter, for orange fluorescence emissions by a 585±42 nm filter and for red fluorescence emissions by a 660±20 nm filter. Debris (non-sperm events) was gated out on the basis of forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. Data were collected from 1 x $10^4$ events and saved as list mode files.

Sperm viability and mitochondrial status were determined by using fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA; green fluorescence)/PI (propidium iodide) dual-staining method and lipophilic cation 5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolylcarbocyanine iodide (JC-1) as described before (Gürler et al., 2016), respectively.

The DCFH is widely used to measure ROS. The diacetate form of DCFH enters the cell and is hydrolyzed by intracellular esterases to liberate DCFH (LeBel et al., 1992). DCFH-

DA is a non-fluorescent agent, which is converted by hydroxyl, nitrogen dioxid radicals, hypochlorous acid, carbonate anion radicals and hydrogen peroxide into the green fluorescent DCFH (Kalyanaraman et al., 2012). For this, the method was performed as described in (Gürler et al., 2016).

CellROX Deep Red probe is a stain for the detection of particularly cellular superoxide anion ($O_2 \cdot -$) and hydroxyl radical (OH•) (Rodrigues, 2015). It fluoresces red (655 nm) due to presence of these oxidation products in cells (Rodrigues, 2015). For the measurement of these oxidation processes in plasma membrane intact sperm, 1 µL of CellROX Deep Red™ (1 mM) and 3 µL of PI (2.99 mM) were added to 496 µL of diluted sperm suspension with Tyrode´s solution.

The fluorescent fatty acid analog C11-BODIPY$^{581/591}$ is used to detect lipid peroxidation (LPO) in the plasma membrane of spermatozoa (Ball and Vo, 2002). The intact probe fluoresces red when intercalated into the membrane and its fluorescence shifts towards green (520nm) emission after attack with oxidative radicals (Brouwers and Gadella, 2003). To quantify the LPO of plasma membrane intact sperm 5 µL of BODIPY C11 (5 mM), and 3 µL of PI (2.99 mM) were added to 492 µL of diluted sperm suspension with Tyrode´s solution.

All sperm samples were incubated at 37 °C for 30 min before flow cytometry. The percentage of plasma membrane and acrosome intact sperm (PMAI), the percentage of sperm with a high mitochondrial membrane potential (HMMP), mean Dichlorofluorescein-diacetate intensity of plasma membrane intact sperm (DCFH), the percentage of plasma membrane intact sperm with a high CellROX Deep Red intensity (CellROX) and mean green fluorescence intensity of sperm (LPO), and were analyzed using CytExpert 1.2 software (Beckman Coulter, Fullerton, CA, USA).

The %DFI values were determined by SCSA™, as described before (Evenson and Jost, 2000). It was performed as previously described in Malama et al. (2017).

### 3.7. Statistical analyses

Mean value and standard deviation (SD) of sperm parameters, depending on treatment conditions and incubation time, were calculated to describe the central tendency and dispersion of the data. Growth curve analysis was used to analyze sperm parameters over the course of a 24 hour incubation period. The relation of the outcome variables (RMS, PMAI, HMMP, DFI%, LPO, DCFH, CellROX) to incubation time was assumed to be linear, quadratic or cubic; thus, time curves were modeled using first-, second- and third-order orthogonal polynomials of incubation time. The fixed effect of *treatment* with sodium pyruvate was added in all time terms. Values of control samples were set as the baseline condition (control vs Pyr-treated sperm) and model parameters were estimated to describe the effect of *treatment* on the rate of change of sperm traits during the 24 hour incubation. Between-ejaculate variability was included as a random effect in a mixed-effects model structure; the intercept of the models was allowed to vary across ejaculates nested within bull. The random effect of the ejaculate (nested within bull) as well as the fixed effects of *treatment*, *incubation time* (time polynomials) and their interaction terms were added individually in a hierarchical order in the model structure. The subsequent improvement of model fit was evaluated by model comparisons based on the -2 log-likelihood ratio criterion (at 0.05 significance level). Parameter-specific P-values were estimated using the normal approximation. The dependent *t* test (at 0.05 significance level) was used for the exploration of differences in sperm parameters between control and Pyr-treated samples at 0h of incubation. Processing of the data, statistical analyses and graphical illustration of the results were carried out using the nlme (Pinheiro et al., 2015), lattice (Sarkar, 2008), and ggplot2 (Wickham, 2009) packages in R version 3.1.3 (R Core Team, 2015).

## 4. Results

Descriptive statistics (mean value±SD) of sperm parameters in relation to the addition of sodium pyruvate and post-thaw incubation time are presented in Table 1. Initial values of sperm traits at 0h did not differ between control and Pyr-treated samples ($P>0.05$ in all cases), with exception of %DFI; control sperm showed higher values of %DFI compared to Pyr-treated sperm [$t(123)=-2.36$, $P<0.05$)].

In overall, Pyr-treated samples showed higher RMS ($b=5.92\pm0.88$, $P<0.0001$) and PMAI ($b=3.57\pm1.18$, $P<0.05$) values as well as lower %DFI values ($b=-14.75\pm0.82$, $P<0.0001$) when compared to control samples (Table 2). On the contrary, the effect of sodium pyruvate addition on the overall values of HMMP, DCFH, LPO and CellROX was not significant ($P>0.05$ for coefficients b in all cases; Table 2).

Sperm traits of control samples were assumed to have a linear, quadratic or cubic relation to incubation time; polynomials of incubation that did not improve the goodness of fit of the applied models ($P>0.05$ for the -2 log-likelihood ratio criterion) were not included in the analysis. The second-order polynomial term of incubation time was the highest one showing a significant relation with RMS, HMMP, DCFH, and LPO of control samples ($P<0.0001$ for the coefficients b of the linear and quadratic term in all cases); this implies that the above mentioned sperm traits responded in a quadratic manner to the increasing duration of incubation. Control samples showed a linear and cubic decrease of PMAI ($P<0.0001$ for the first-order polynomial of incubation) and CellROX ($P<0.001$ for the first-, second- and third-order polynomial) during the 24 hour incubation, respectively (Table 2). %DFI of

control samples increased in a cubic manner with increasing incubation time ($P<0.0001$ for the first-, second- and third-order polynomial; Table 2).

Differences in the change of sperm parameters over time between control and Pyr-enriched group were expressed as an interaction of *treatment group* × *polynomial term of incubation*; estimated coefficients b and the respective P values are presented in Table 2. The mean values (±SEM) of the experimental data and the model-predicted time curves of RMS, PMAI, HMMP, DCFH, LPO, CellROX and DFI% for control and Pyr-treated samples are demonstrated in Figures 1 to 7, respectively. Pyr-treated samples showed a slower decrease of RMS and HMMP compared to control samples ($P<0.0001$ for the effect of treatment on the quadratic polynomial of incubation in both cases; Table 2). Moreover, the addition of sodium pyruvate had a beneficial effect on the rate of change of PMAI and %DFI values ($P<0.0001$ for the effect of treatment on the linear and quadratic polynomial of incubation, respectively; Table 2). While Pyr-treated samples showed a slower decrease of CellROX values ($P<0.0001$ for the effect of treatment on the first-, second- and third-order polynomial of incubation; Table 2) compared to control samples, the rate of change of DCFH and LPO during the 24 hour incubation did not differ between the two groups ($P>0.05$ in all cases; Table 2).

## 5. Discussion

The results of our study showed that values of RMS, PMAI, and HMMP were higher in the Pyr treated group compared to the control group in overall. Furthermore, the addition of sodium pyruvate led to a slower decrease in RMS, PMAI, and HMMP during the 24 hour incubation time. In this respect, our data not only verified the positive effects of the addition of sodium pyruvate to the semen extender on sperm quality parameters as reported before

(Bilodeau et al., 2002; Breininger and Beconi, 2014; Upreti et al., 1998), but also indicate that effect of sodium pyruvate can be visible after 24-hour incubation at 37° C in cryopreserved bovine sperm.

It is known that exogenous pyruvate increases the mitochondrial activity and post-thaw motility of sperm (Darr et al., 2016; Hereng et al., 2011). Additionally, antioxidant effect of sodium pyruvate has been described before (Bilodeau et al., 2002; Breininger and Beconi, 2014; Gibb et al., 2015; Upreti et al., 1998). Therefore, the values of RMS, PMAI and HMMP and their close relationships between each other in both groups could be explained by increasing of ATP production with the presence of sodium pyruvate in semen extender. The pathway of ATP production, which is utilizing during sperm function and motility, either glycolysis or OXPHOS, is unclear yet (du Plessis et al., 2015; Nascimento et al., 2008; Tourmente et al., 2015). While glycolysis occurs in the head and fibrous sheath of the flagellum of sperm, OXPHOS occurs in the neck of the sperm where the mitochondria are located (Westhoff and Kamp, 1997). The RMS, PMAI, and HMMP values of the Pyr-treated group did not differ at 0h after thawing compared to the control group. However, our results show that during a 24- hour incubation period at 37° C, values for RMS, PMAI and HMMP were higher in the Pyr-treated group than in the control group, which were almost zero. Numerous studies suggested that OXPHOS is the primary source of ATP production for sperm function and motility in several mammalian species e.g. stallion (Darr et al., 2016; Gibb et al., 2014), mouse (Tourmente et al., 2015) and boar (Guo et al., 2017). The higher motility values in the Pyr-treated group during the 24h incubation time indicate that exogenous pyruvate was consumed using the OXPHOS pathway in the sperm mitochondria. The hypothesis that pyruvate is used by OXPHOS in bovine sperm is also supported by higher HMMP and CellROX values that indicate higher mitochondrial activity in the Pyr-treated group. Therefore, we can speculate that, after a prolonged incubation time of frozen

thawed bovine sperm, OXPHOS plays a more important role on sperm function and motility than glycolysis. In line to the current literature in other mammalian species (Darr et al., 2016; Gibb et al., 2014; Guo et al., 2017; Tourmente et al., 2015) we can suggest that exogenous pyruvate as an energy source improves sperm motility as well as plasma membrane and acrosomal integrity and mitochondrial membrane potential during 24h incubation in frozen thawed bovine sperm by inducing ATP production utilizing the OXPHOS pathway in sperm mitochondria.

According to our results, DCFH, CellROX and LPO values were affected after thawing in sperm of both groups. An increase in ROS synthesis after the freezing and thawing processes has been reported in several studies (Bailey et al., 2000; Bansal and Bilaspuri, 2010; Gürler et al., 2016). This is also in accordance with our results that showed a rise of DCFH and CellROX levels during the incubation of cryopreserved sperm after thawing. Sperm contain a relatively high amount of polyunsaturated fatty acids (PUFAs) in the plasma membrane (Agarwal et al., 2014). These PUFAs increase the sensitivity to oxidative stress (Ball et al., 2001; Maneesh and Jayalekshmi, 2006), which could explain the increase of LPO during the incubation of frozen thawed sperm. As sodium pyruvate has a scavenging effect on $H_2O_2$ (Bilodeau et al., 2002; Breininger and Beconi, 2014; Giandomenico et al., 1997; Melzer and Schmidt, 1988; O'Donnell-Tormey et al., 1987; Salahudeen et al., 1991; Upreti et al., 1998), it is initially surprising that DCFH values which are monitoring ROS levels did not differ between Pyr-treated and control groups. The reason for this unexpected phenomenon might be the low specifity of the DCFH probe to detect $H_2O_2$ (Kalyanaraman et al., 2012). Although some studies use DCFH as a probe to measure $H_2O_2$ abundancy (Gomes et al., 2005; Gürler et al., 2016), de Castro et al. (2016) could not detect differences in DCFH fluorescence intensity of bovine sperm after addition of $H_2O_2$ in concentrations up to 200µM. Kalyanamaran et al. (2012) reported that DCFH reacts not only with $H_2O_2$, but also with other

ROS species including hydroxyl radicals (•OH), nitrogen dioxid radicals ($•NO_2$), hypochlorous acid (HOCl) and carbonate anion radicals ($CO_3•-$). As described above sodium pyruvate has positive effects on metabolism of mitochondria. In a series of studies (Boveris et al., 2006; Korshunov et al., 1997; Starkov and Fiskum, 2003) it was demonstrated that a high metabolism is associated with high concentrations of ROS. Therefore, the inhibition of $H_2O_2$ synthesis (Bilodeau et al., 2002; Breininger and Beconi, 2014; O'Donnell-Tormey et al., 1987; Upreti et al., 1998) by sodium pyruvate could superimposed by its stimulating effect on ROS synthesis. The high CellROX values measured in our study in the Pyr-treated group also support this hypothesis. To the best of our knowledge, there is only one published preliminary report using of CellROX Deep Red in bovine sperm (Bianchi-Alves et al., 2015). The authors claimed that CellROX Deep Red could be used to identify ROS generation in bovine spermatozoa. Davila et al. (2015) performed an investigation on equine sperm and came to the conclusion that CellROX Deep Red is an indicator especially for superoxide anion synthesized especially by mitochondria. Furthermore Darr et al. (2016) noticed that the addition of pyruvate to the incubation medium increased the mitochondrial activity as well as the superoxide anion level in equine sperm. The results of our study are in agreement with the last mentioned studies.

The rise of the LPO during the incubation period did not differ between the Pyr treated and control groups. Also Gibb et al. (Gibb et al., 2014) could not find an effect of 10mM pyruvate on LPO levels in fresh stallion semen using the flow cytometric anti-4-hydroxynonenal (anti-4HNE) assay. Breininger and Beconi (Breininger and Beconi, 2014), however, demonstrated that the addition of sodium pyruvate to the extender in cryopreserved boar semen decreased LPO levels compared to control samples. They used 0, 5, 10 mM sodium pyruvate and quantified LPO by measuring the concentration of thiobarbituric acid reactive substances (TBARS) using a spectrofluorometer. Differences in the sensitivity to

LPO between species because of variable amounts of PUFAs in the plasma membrane of sperm (Ahluwalia and Holman, 1969) and differences in the sensitivity between assays to detect LPO (Domínguez-Rebolledo et al., 2010) might be responsible for these discrepancies between the studies.

As far as we know, up to now no other study has been performed to investigate the effects of sodium pyruvate on DNA integrity of cryopreserved bovine sperm. The results of our study revealed a positive effect of sodium pyruvate on sperm nuclear DNA integrity in cryopreserved sperm. A rise in DNA damage induced by freezing and thawing processes has been reported in trout (Labbe et al., 2001), canine (Kim et al., 2010), human (Kalthur et al., 2008), equine (Ball et al., 2001) and bovine (Waterhouse et al., 2010) sperm and it is well known that DNA integrity plays an important role for the development of the pre-implantation embryo (Aitken et al., 2009; Evenson, 2016). Hydrogen peroxide is a ROS that causes DNA lesions (De Castro et al., 2016; Villani et al., 2010) and it is described as the primary source of DNA damage after cryopreservation of bovine sperm (Gürler et al., 2016). The inhibiting effect of sodium pyruvate on $H_2O_2$ (Bilodeau et al., 2002) might be responsible for the lower DNA damage of Pyr-treated sperm. This result indicates, that increased synthesis of other ROS (especially superoxide anion and hydroxyl radical) in cryopreserved sperm has a less severe damaging effect on DNA integrity compared to hydrogen peroxide.

Overall, the addition of antioxidants is not able to inhibit all types of ROS, but they have specific effects on different ROS and their generating pathways. According to our results, sodium pyruvate is for example an antioxidant inhibiting especially the synthesis of $H_2O_2$, which damages DNA of sperm. Therefore, it protects the DNA of cryopreserved sperm (Gürler et al., 2016). On the other hand side, pyruvate is an energy source and increases the mitochondrial activity that can be the source of other ROS such superoxide anion and hydroxyl radical. Future studies should not only focus on the synthesis of the total amount of

ROS, but of specific ROS induced by several sperm treatments to develop methods to inhibit especially those with damaging effects on sperm by addition of specific antioxidants. However, one should be clear that some antioxidants like pyruvate also play other roles in the cell metabolism important for fertility of sperm.

In conclusion, the results of our study show that the addition of sodium pyruvate improved motility, plasma membrane and acrosome integrity, mitochondrial membrane potential and DNA integrity of cryopreserved bovine sperm and had no effects on lipid peroxidation, although it stimulated the synthesis of some ROS.

## 6. Tables

**Table 1**: Changes in viability (RMS, PMAI, HMMP), ROS synthesis (DCFH, CellROX Deep Red), lipid peroxidation (LPO) and DNA integrity (%DFI) of sperm cryopreserved in Triladyl® extender without (control) and with 5 mM sodium pyruvate (Pyr) 0, 3, 6, 12 and 24h after thawing and incubation at 37° C. Values are means ± SD of 69 ejaculates from 23 bulls (3 ejaculates from each bull).

| | | Incubation times (hours) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 3 | 6 | 12 | 24 |
| RMS (%) | Pyr (+) | 32.29±9.17 | 30.87±9.32 | 28.44±9.48 | 16.5±9.24 | 0.35±0.4 |
| | control | 35.21±9.97 | 31.61±12.53 | 11.76±13.09 | 0.34±0.53 | 0.26±0.44 |
| PMAI (%) | Pyr (+) | 53.32±9.66 | 46.78±9.79 | 42.35±9.92 | 34.44±10.61 | 12.17±7.35 |
| | control | 55.82±9.06 | 49.95±8.86 | 46.25±10.89 | 18.43±18.2 | 0.93±0.46 |
| HMMP (%) | Pyr (+) | 59.54±11.28 | 52.1±11.87 | 44.38±11.79 | 33.45±11.62 | 5.26±5.35 |
| | control | 62.65±11.1 | 54.45±13.28 | 45.8±14.95 | 12.98±17.64 | 0.83±1.28 |
| DCFH (channels) | Pyr (+) | 12189±4728 | 13171±4453 | 17339±7870 | 18536±9468 | 20467±9032 |
| | control | 12540±4236 | 13587±4656 | 17975±8398 | 19876±11009 | 21673±10650 |
| CellROX Deep Red (%) | Pyr (+) | 47.05±11.10 | 43.11±10.62 | 38.55±11.15 | 29.78±9.87 | 5.91±6.05 |
| | control | 50.69±10.59 | 46.44±10.72 | 41.66±11.32 | 14.44±16.74 | 0.39±1.08 |
| LPO (channels) | Pyr (+) | 21809±6630 | 27266±4248 | 30995±4527 | 33983±5393 | 38787±6842 |
| | control | 21746±5304 | 26302±4443 | 32449±4005 | 35843±4888 | 40222±7037 |
| % DFI (%) | Pyr (+) | 9.27±4.07 | 12.36±5.82 | 17.47±10.11 | 38.96±13.49 | 50.06±10.60 |
| | control | 11.22±5.49 | 21.34±11.1 | 40.4±15.28 | 61.12±13.82 | 65.87±9.84 |

Rapidly motile sperm (RMS), plasma membrane and acrosome intact sperm (PMAI), sperm with a high mitochondrial membrane potential (HMMP), Dichlorofluorescein intensity of plama membrane intact sperm (DCFH), CellROX Deep Red intensity of plama membrane intact sperm (CellROX Deep Red), Lipid Peroxidation (LPO), percentage of sperm with a high DNA fragmentation index (%DFI).

**Table 2:** Estimated coefficients b (mean value±SEM) and the parameter-specific P values that describe the effect of experimental conditions (polynomial term of incubation time, addition of sodium pyruvate and their interaction term) on viability (RMS, PMAI, HMMP), ROS synthesis (DCFH, CellROX Deep Red), lipid peroxidation (LPO) and DNA integrity (%DFI) of sperm cryopreserved in Triladyl® extender without (control) and with 5 mM sodium pyruvate (Pyr) 0, 3, 6, 12 and 24h after thawing and incubation at 37° C.

| | RMS | PMAI | HMMP | DCFH | CellROX Deep Red | LPO | DFI % |
|---|---|---|---|---|---|---|---|
| (Intercept) | 15.88±1.15 (P<0.0001) | 34.40±1.35 (P<0.0001) | 35.46±1.58 (P<0.0001) | 16756.55±678.71 (P<0.0001) | 30.72±1.25 (P<0.0001) | 31012.35±395.51 (P<0.0001) | 40.08±0.86 (P<0.0001) |
| Time | -333.64±11.44 (P<0.0001) | -540.37±12.99 (P<0.0001) | -605.86±14.15 (P<0.0001) | 141832.10±5986 (P<0.0001) | -503.72±10.93 (P<0.0001) | 148917±4652.91 (P<0.0001) | 519.48±13.15 (P<0.0001) |
| Time² | 191.39±11.44 (P<0.0001) | na | 147.49±14.15 (P<0.0001) | -30675.29±4744.51 (P<0.0001) | 72.15±10.93 (P<0.0001) | -52032.37±4653.60 (P<0.0001) | -253.08±13.15 (P<0.0001) |
| Time³ | 32.08±11.44 (P>0.05) | na | na | na | 106.31±10.93 (P<0.0001) | na | -58.56±13.15 (P<0.001) |
| Condition: Pyr (+) | 5.92±0.88 (P<0.0001) | 3.57±1.18 (P<0.05) | 3.70±1.35 (P>0.05) | na | 2.29±1.34 (P>0.05) | na | -14.75±0.82 (P<0.0001) |
| Time x Pyr (+) | 20.10±13.51 (P>0.05) | 167.85±15.11 (P<0.0001) | 109.12±16.29 (P<0.0001) | na | 118.61±13.45 (P<0.0001) | na | -108.47±16.82 (P<0.0001) |
| Time² x Pyr (+) | -207.92±13.51 (P<0.0001) | na | -149.49±16.29 (P<0.0001) | na | -104.02±13.45 (P<0.0001) | na | 189.41±16.82 (P<0.0001) |
| Time³ x Pyr (+) | 4.42±13.51 (P>0.05) | na | na | na | -111.65±13.45 (P<0.0001) | na | -34.11±16.82 (P>0.05) |

Rapidly motile sperm (RMS), Plasma membrane and acrosome intact (PMAI), High mitochondrial membrane potential (HMMP), Dichlorfluorescein (DCFH), CellROX Deep Red (CellROX Deep Red), Lipid Peroxidation (LPO), DNA fragmentation index (%DFI) *na: term not included in the structure of the final model.*

## 7. Figures

**Fig. 1**: Mean values (±SEM) and model-predicted time curves of sperm parameters (RMS, PMAI, HMMP, DCFH) in cryopreserved bovine sperm diluted with Triladyl® extender without (continuous lines) and with the addition of sodium pyruvate (dashed lines). In each of 23 bulls 3 ejaculates were examined. Rapidly motile sperm (RMS), plasma membrane and acrosome intact sperm (PMAI), sperm with a high mitochondrial membrane potential (HMMP), Dichlorfluorescein (DCFH).

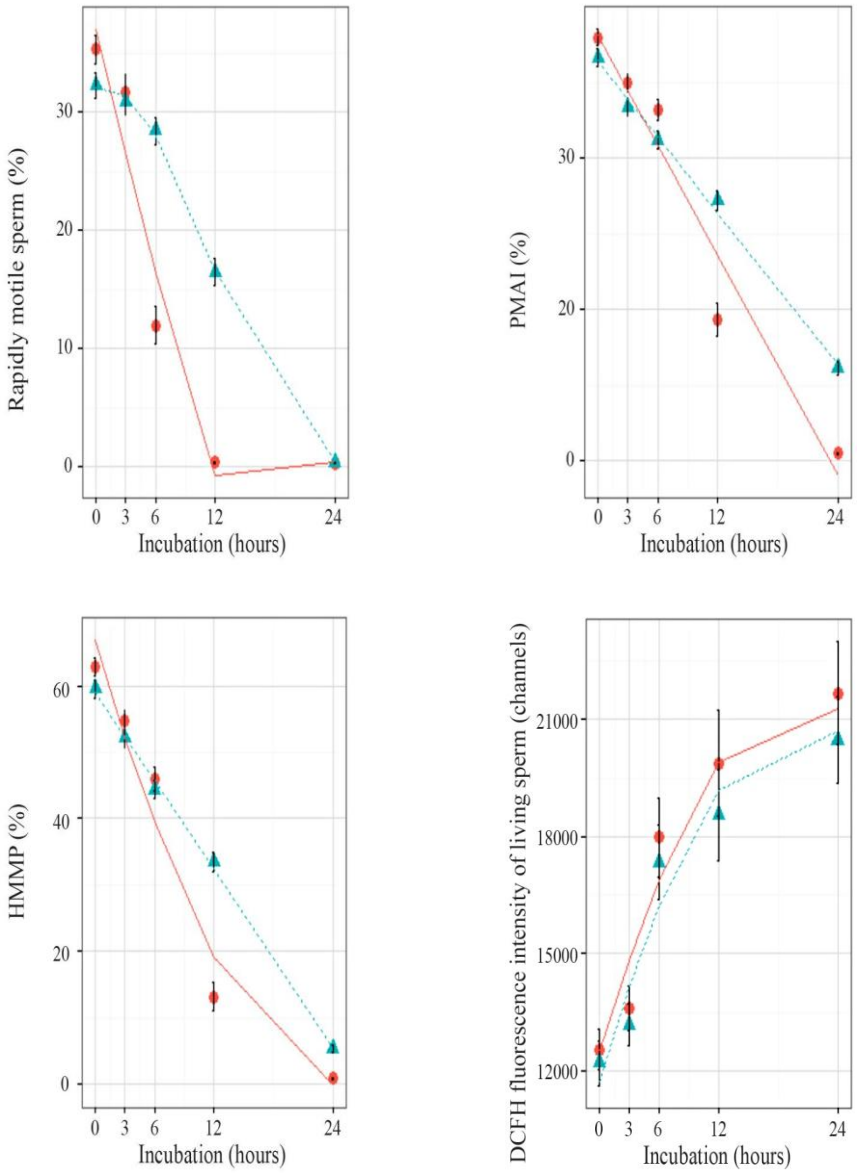
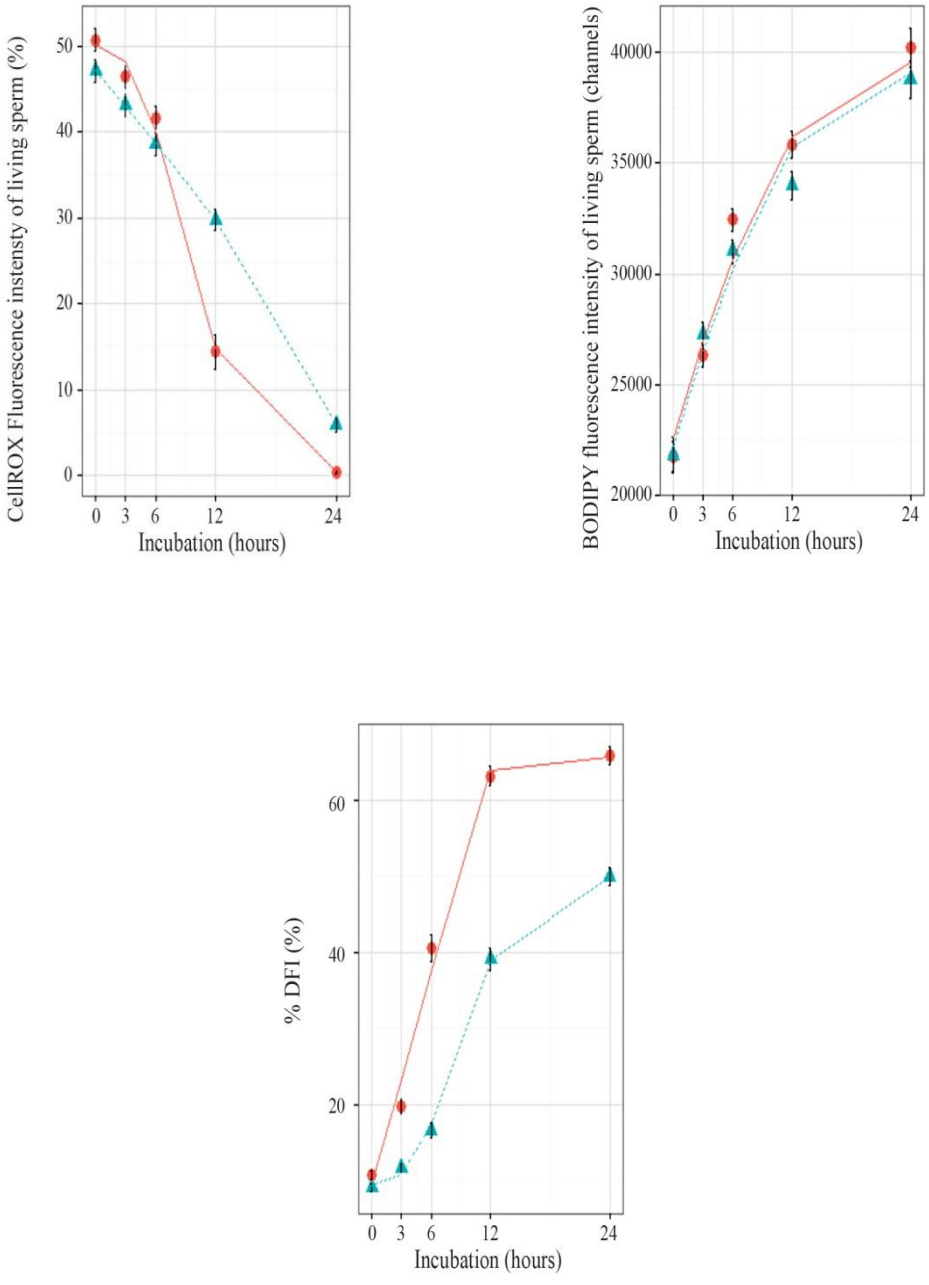


**Fig. 2**: Mean values (±SEM) and model-predicted time curves of sperm parameters (CellROX, LPO, DFI) in cryopreserved bovine sperm diluted within Triladyl® extender without (continuous lines) and with the addition of sodium pyruvate (dashed lines). In each of 23 bulls 3 ejaculate were examined. CellROX Deep Red (CellROX), BODIPY (Lipid peroxidation (LPO)), DNA fragmentation index (DFI).



## 8. References


Agarwal, A., Virk, G., Ong, C., Plessis, S.S., 2014. Effect of Oxidative Stress on Male Reproduction. The World Journal of Men´s Health. 32, 1–17.

Ahluwalia, B., Holman, R.T., 1969. Fatty acid composition of lipids of bull, boar, rabbit and human semen. J. Reprod. Fertil. 18, 431–437.

Aitken, R.J., De Iuliis, G., McLachlan, R., 2009. Biological and clinical significance of DNA damage in the male germ line. Int J Androl. 32, 46–56.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., Irvine, D.S., 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol. Reprod. 59, 1037–1046.

Bailey, J.L., Bilodeau, J.F., Cormier, N., 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. J. Androl. 21, 1,7.

Ball, B.A., Vo, A.T., Baumber, J., 2001. Generation of reactive oxygen species by equine spermatozoa. Am. J. Vet. Res. 62, 508–515.

Ball, B.A., Vo, A.T., 2002. Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C11-BODIPY581/591. J. Androl. 23, 259–269.

Bansal, A.K., Bilaspuri, G.S., 2010. Impacts of oxidative stress and antioxidants on semen functions. Vet. Med. Int. 2011, 686137.

Bianchi-Alves, M.R. , Pinto, S.C.C., Almeida, D.S., Arruda, R.P., Andrade, A.F.C. , Celeghini, E.C.C., 2015. Cellrox Deep Red ® is effective for detecting oxidative stress in bovine sperm : preliminary studies. Anim. Reprod. 12, 542.

Bilodeau, J.F., Blanchette, S., Cormier, N., Sirard, M.A., 2002. Reactive oxygen species-

mediated loss of bovine sperm motility in egg yolk Tris extender: Protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology 57, 1105–1122.

Bollwein, H., Fuchs, I., Koess, C., 2008. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. Reprod Domest Anim 43, 189–195.

Boveris, A., Valdez, L.B., Zaobornyj, T., Bustamante, J., 2006. Mitochondrial metabolic states regulate nitric oxide and hydrogen peroxide diffusion to the cytosol. Biochim. Biophys. Acta - Bioenerg. 1757, 535–542.

Breininger, E., Beconi, M.T., 2014. Ascorbic acid or pyruvate counteracts peroxidative damage in boar sperm cryopreserved with or without α -tocopherol. Anim. Sci. Pap. Reports 32, 15–23.

Brouwers, J.F.H.M., Gadella, B.M., 2003. In situ detection and localization of lipid peroxidation in individual bovine sperm cells. Free Radic. Biol. Med. 35, 1382–1391.

Bucak, M.N., Tuncer, P.B., Sarıözkan, S., Başpinar, N., Taşpinar, M., Çoyan, K., Bilgili, A., Akalin, P.P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroğlu, A., Öztuna, D., 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage. Cryobiology 61, 248–253.

Darr, C.R., Varner, D.D., Teague, S., Cortopassi, G.A., Datta, S., Meyers, S.A., 2016. Lactate and Pyruvate Are Major Sources of Energy for Stallion Sperm with Dose Effects on Mitochondrial Function, Motility, and ROS Production. Biol. Reprod. 95, 1–11.

Davila, M.P., Muñoz, P.M., Tapia, J.A., Ferrusola, C.O., Da Silva C, C.B., Peña, F.J., 2015. Inhibition of mitochondrial complex I leads to decreased motility and membrane

integrity related to increased hydrogen peroxide and reduced ATP production, while the inhibition of glycolysis has less impact on sperm motility. PLoS One 10, 1–21.

De Castro, L.S., De Assis, P.M., Siqueira, A.F.P., Hamilton, T.R.S., Mendes, C.M., Losano, J.D.A., Nichi, M., Visintin, J.A., Assumpção, M.E.O.A., 2016. Sperm oxidative stress is detrimental to embryo development: A dose-dependent study model and a new and more sensitive oxidative status evaluation. Oxid. Med. Cell. Longev. 2016.

Domínguez-Rebolledo, Á., Martínez-Pastor, F., Fernández-Santos, M.R., Del Olmo, E., Bisbal, A., Ros-Santaella, J.L., Garde, J.J., 2010. Comparison of the TBARS Assay and BODIPY C11 Probes for Assessing Lipid Peroxidation in Red Deer Spermatozoa. Reprod. Domest. Anim. 45, 360–368.

du Plessis, S.S., Agarwal, A., Mohanty, G., van der Linde, M., 2015. Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? Asian J. Androl. 17, 230–5.

Evenson, D., Jost, L., 2000. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci. 169–189.

Evenson, D.P., 2016. The Sperm Chromatin Structure Assay ( SCSA ® ) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility 169, 56–75.

Giandomenico, A.R., Cerniglia, G.E., Biaglow, J.E., Stevens, C.W., Koch, C.J., 1997. The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide. Free Radic. Biol. Med. 23, 426–434.

Gibb, Z., Lambourne, S.R., Aitken, R.J., 2014. The Paradoxical Relationship Between Stallion Fertility and Oxidative Stress. Biol. Reprod. 91, 1–10.

Gibb, Z., Lambourne, S.R., Aitken, R.J., 2014. Pyruvate and L-carnitine are pro-survival factors for stallion spermatozoa. J. Equine Vet. Sci. 34, 31–32.

Gibb, Z., Lambourne, S.R., Quadrelli, J., Smith, N.D., Aitken, R.J., 2015. L-Carnitine and Pyruvate Are Prosurvival Factors During the Storage of Stallion Spermatozoa at Room Temperature. Biol. Reprod. 93, 104.

Gomes, A., Fernandes, E., Lima, J.L.F.C., 2005. Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45–80.

Guo, H., Gong, Y., He, B., Zhao, R., 2017. Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. Theriogenology 87, 276–283.

Gürler, H., Malama, E., Heppelmann, M., Calisici, O., Leiding, C., Kastelic, J.P., Bollwein, H., 2016. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. Theriogenology 86, 562–571.

Hereng, T.H., Elgstøeen, K.B.P., Cederkvist, F.H., Eide, L., Jahnsen, T., Sklhegg, B.S., Rosendal, K.R., 2011. Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. Hum. Reprod. 26, 3249–3263.

Januskauskas, A., Johannisson, A., Rodriguez-Martinez, H., 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60, 743–758.

Jenkins, F.J., Van Houten, B., Bovbjerg, D.H., 2014. Effects on DNA damage and/or repair processes as biological mechanisms linking psychological stress to cancer risk. J. Appl. Biobehav. Res. 19, 3–23.

Kalthur, G., Adiga, S.K., Upadhya, D., Rao, S., Kumar, P., 2008. Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. Fertil. Steril. 89, 1723–1727.

Kalyanaraman, B., Darley-Usmar, V., Davies, K.J.A., Dennery, P.A., Forman, H.J., Grisham, M.B., Mann, G.E., Moore, K., Roberts, L.J., Ischiropoulos, H., 2012. Measuring reactive oxygen and nitrogen species with fluorescent probes: Challenges and limitations. Free Radic. Biol. Med. 52, 1–6.

Kim, S.H., Yu, D.H., Kim, Y.J., 2010. Effects of cryopreservation on phosphatidylserine translocation, intracellular hydrogen peroxide, and DNA integrity in canine sperm. Theriogenology 73, 282–292.

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., Aitken, R.J., 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J. Clin. Endocrinol. Metab. 93, 3199–3207.

Korshunov, S.S., Skulachev, V.P., Starkov, A.A., 1997. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett. 416, 15–18.

Labbe, C., Martoriati, A., Devaux, A., Maisse, G., 2001. Effect of sperm cryopreservation on sperm DNA stability and progeny development in rainbow trout. Mol. Reprod. Dev. 60, 397–404.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Lushchak, V.I., 2014. Classification of oxidative stress based on its intensity. EXCLI J. 13, 922–937.

Maneesh, M., Jayalekshmi, H., 2006. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian J. Clin. Biochem. 21, 80–9.

Melzer, E., Schmidt, H.L., 1988. Carbon isotope effects on the decarboxylation of carboxylic acid. Biochem. J. 252, 913–915.

Nascimento, J.M., Shi, L.Z., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E.L., Berns, M.W., 2008. Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. J. Cell. Physiol. 217, 745–751.

O'Donnell-Tormey, J., Nathan, C.F., Lanks, K., DeBoer, C.J., de la Harpe, J., 1987. Secretion of pyruvate. An antioxidant defense of mammalian cells. J. Exp. Med. 165, 500–14.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2015. nlme: Linear and non linear mixed-effects models R package version 3.1-120. http://cran.r-project.org/package=nlme

Rao, T.K.S., Kumar, N., Patel, N.B., Chauhan, I., Chaurasia, S., 2013. Sperm selection techniques and antioxidant fortification in low grade semen of bulls: Review. Vet. World 6, 579–585.

Rodrigues, M.B., 2015. An Efficient Technique to Detect Sperm Reactive Oxygen Species: The CellRox Deep Red® Fluorescent Probe. Biochem. Physiol. Open Access 4, 0–5.

Rueff, J., Brás, A., Cristóvão, L., Mexia, J., Sáda Costa, M., Pires, V., 1993. DNA strand breaks and chromosomal aberrations induced by H2O2 and 6°Co y-radiation. Mutat. Res. - Fundam. Mol. Mech. Mutagen. 289, 197–204.

Salahudeen, A.K., Clark, E.C, Nath, A.K., 1991. Hydrogen peroxide-induced renal injury: A protective role for pyruvate in vitro and in vivo. J. Clin. Invest. 88, 1886–1893.

Sarkar, D., 2008. lattice: Multivariate Data Visualization with R. Springer, New York.

Starkov, A.A., Fiskum, G., 2003. Regulation of brain mitochondrial H2O2 production by membrane potential and NAD(P)H redox state. J. Neurochem 86, 1101–1107.

Tardif, A.L., Farrell, P.B., Trouern-Trend, V., Simkin, M.E., Foote, R.H., 1998. Use of Hoechst 33342 stain to evaluate live fresh and frozen bull sperm by computer-assisted analysis. J. Androl. 19, 201–206.

R Core Team, 2015. R: A language and environment for statistical computing R Foundation for Statistical Computing. Vienna, Austria.

Thomas, C.A., 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. Biol. Reprod. 58, 786–793.

Tirpák, F., Slanina, T., Ofúkaný, M., Lukáč, N., Massányi, P., 2015. Effect of Taurine on Bovine Spermatozoa Motility Parameters Following Cryopreservation. Slovak J. Anim. Sci. 48, 49–56.

Tourmente, M., Villar-Moya, P., Rial, E., Roldan, E.R.S., 2015. Differences in ATP generation via glycolysis and oxidative phosphorylation and relationships with sperm motility in mouse species. J. Biol. Chem. 290, 20613–20626.

Upreti, G.C., Jensen, K., Munday, R., Duganzich, D.M., Vishwanath, R., Smith, J.F., 1998. Studies on aromatic amino acid oxidase activity in ram spermatozoa: Role of pyruvate as an antioxidant. Anim. Reprod. Sci. 51, 275–287.

Villani, P., Eleuteri, P., Grollino, M.G., Rescia, M., Altavista, P., Spanò, M., Pacchierotti, F., Cordelli, E., 2010. Sperm DNA fragmentation induced by DNAse I and hydrogen peroxide: An in vitro comparative study among different mammalian species. Reproduction 140, 445–452.

Ward, J.F., Blakely, W.F., Joner, E.I., 1985. Mammalian cells are not killed by DNA single-

strand breaks caused by hydroxyl radicals from hydrogen peroxide. Radiat. Res. 103, 383–392.

Waterhouse, K.E., Gjeldnes, a., Tverdal, a., De Angelis, P.M., Farstad, W., Håård, M., Kommisrud, E., 2010. Alterations of sperm DNA integrity during cryopreservation procedure and in vitro incubation of bull semen. Anim. Reprod. Sci. 117, 34–42.

Westhoff, D., Kamp, G., 1997. Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa. J. Cell Sci. 110, 1821–1829.

Wickham, H., 2009. ggplot2: Elegant graphics for data analysis. Springer, New York.

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